The FASEB Journal express article 10.1096/fj.01-0736fje. Published online January 30, 2002.

Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish

Ju Hyun Cho*, In Yup Park*, Hun Sik Kim*, Won Taek Lee[†], Mi Sun Kim [‡], and Sun Chang Kim*

*Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea; [†]Department of Anatomy, Yonsei University College of Medicine, Seoul 120-752, Korea; and [‡]Biomass Team, Korea Institute of Energy Research, Taejon 305-343, Korea

Corresponding author: Sun Chang Kim, Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1 Kusong-dong Yusong-gu, Taejon 305-701, Korea. E-mail: sckim@mail.kaist.ac.kr

ABSTRACT

Parasin I is a potent 19-residue antimicrobial peptide isolated from the skin mucus of wounded catfish (*Parasilurus asotus*). Here we describe the mechanism of parasin I production from histone H2A in catfish skin mucosa on epidermal injury. Cathepsin D is found to exist in the mucus as an inactive proenzyme (procathepsin D), and a metalloprotease, induced on injury, cleaves procathepsin D to generate active cathepsin D. This activated form of cathepsin D then cleaves the Ser^{19}–Arg^{20} bond of histone H2A to produce parasin I. Immunohistochemical analysis reveals that unacetylated histone H2A, a precursor of parasin I, and procathepsin D are present in the cytoplasm of epithelial mucous cells and that parasin I is produced on the mucosal surface on epidermal injury. Western blot analysis shows that parasin I is also present in the skin mucus of other fish species. Furthermore, parasin I shows good antimicrobial activity against fish-specific bacterial pathogens. Taken together, these results indicate that cathepsin D and a metalloprotease participate in the production of parasin I from histone H2A and that parasin I contributes to the innate host defense of the fish against invading microorganisms.

Key words: innate immunity • metalloprotease • *Parasilurus asotus* • teleost

Antibody and specific cell-mediated immune responses of lower vertebrates such as teleost fish are significantly less sophisticated than those of higher animals (1). In fish, the immune response is also limited in response time by the temperature constraints on fish metabolism (2). Therefore, fish rely heavily on innate or nonspecific immune mechanisms for initial protection against infectious agents. Antimicrobial peptides have been isolated from a multitude of animals and plant species (3), including fish (4–6), and are recognized as important components of the nonspecific defense system (7). These peptides can function intracellularly, as in circulating leukocytes, or in the external environment after release by secretory cells and other granulated epithelia (8). Recent studies have focused on mucus-derived antimicrobial peptides and their roles in innate host defense at organism-environment

interfaces, such as the integument and the respiratory and digestive epithelia (9). Biological studies of these antimicrobial peptides have demonstrated that in addition to killing microorganisms, some of these peptides function in regulating cell proliferation (10), extracellular matrix production (11), and cellular immune responses (12). Thus, they provide a powerful defense system that can both protect the mucosal surfaces from infection and signal host cells to change their behavior in response to external injury.

Recently, a potent 19-residue linear antimicrobial peptide named parasin I was isolated from the skin mucus of wounded catfish (5). The amino acid sequence of parasin I (KGRGKQGGKVRAKAKTRSS) is identical at 17 of 19 residues to the N-terminal region of histone H2A from calf thymus (13). The function of histones historically has been viewed mainly in connection with DNA stabilization and gene expression. However, there is growing evidence that histones may be involved in a multitude of biological functions, including host defense (6, 14–17). Histones have been detected in extracellular fluid as well as on the surface of intact cells, and the proteolytic processing of histone H2A to yield an antimicrobial peptide buforin I was reported (17). Buforin I is a 39-residue toad peptide produced from histone H2A by pepsin action (17). The high degree of homology between parasin I and the N-terminal region of histone H2A suggests that parasin I may be generated by proteolytic cleavage of histone H2A. However, the protease responsible for the generation of parasin I from histone H2A has not yet been identified. Furthermore, the observation that parasin I is found only in the skin mucus of wounded catfish and not in unwounded catfish (5) suggests the involvement of an inducible mechanism in parasin I production. In this study, we describe the mechanism for the generation of parasin I from histone H2A in catfish skin mucosa. We also assess the biological role of parasin I in the innate host defense of fish against invading microorganisms.

MATERIALS AND METHODS

Preparation of mucus extracts

Catfish (400–500 g each, provided from natural pounds near Taejon, Korea) were kept for 1 wk in the laboratory in a 70-liter tank. We wounded catfish by scratching the skin with sandpaper and subsequently stunning them by electroshock 5 h after the wounding. We collected mucus samples (50 mg of protein) from normal (unwounded) and wounded catfishes by lightly scraping the surface of the dorso-lateral parts of the skin with a plastic spatula. Ventral skin mucus was not collected so as to avoid possible contamination with intestinal contents. The collected mucus was homogenized in 100 ml of 50 mM ammonium acetate (pH 7.0) containing 15% (v/v) glycerol. After centrifugation at 12,000 g for 40 min at 4°C, the supernatant was collected, and these mucus extracts were used as the source for the purification of proteases.

Assay for proteolytic activity

We examined proteolytic cleavage of histone H2A by incubating 10 μg of unacetylated calf thymus histone H2A (Roche, Manheim, Germany) with mucus extracts (5 μg of protein) from either wounded catfish or normal catfish in reaction buffers ranging in pH from 3.0 to 10.0 (total reaction volume, 50 μl). After incubation for 1 h at room temperature, the reactions were stopped by boiling for 5 min and analyzed by 16.5% tricine sodium dodecylsulfate polyacrylamide gel

electrophoresis (SDS-PAGE). For protease inhibition assays, we pre-incubated the mucus extracts (5 μg of protein) for 1 h at room temperature with each of the following protease inhibitors: phenylmethylsulfonyl fluoride (PMSF) (10 mM), E-64 (50 μM), pepstatin A (50 μM), and ethylenediaminetetraacetic acid (EDTA) (5 mM). Histone H2A (10 μg) was then added, and the protease reactions were allowed to proceed for an additional hour (final reaction volume, 50 μl in reaction buffer).

Purification of a specific protease from the mucus of wounded and normal catfishes

All purification steps were carried out at 4°C. Mucus extracts of wounded catfish that contained ~50 mg of protein (5 mg/ml) were acidified to pH 3.5 and loaded into a column of pepstatin A-agarose equilibrated with 50 mM sodium acetate (pH 3.5). The column was washed with the same buffer at a flow rate of 0.2 ml/min until the absorbance at 280 nm fell to a baseline. This pepstatin A flow-through fraction was concentrated and desalted by using a Centricon microconcentrator (Amicon, Beverly, MA) with a 10 kDa molecular mass cut-off membrane for further use. Proteins of interest were then eluted from the pepstatin A-agarose column loaded with the mucus extracts at the same flow rate with 50 mM Tris-HCl (pH 8.5) containing 0.5 M NaCl. Fractions (1 ml) were collected and analyzed by 16.5% tricine SDS-PAGE for parasin I-producing activity at pH 6.0. Active fractions were concentrated and desalted as above. The pooled active fractions from the pepstatin A-agarose step were loaded into a Mono Q (HR 5/5) column (0.5×5.0 cm, Pharmacia LKB, Uppsala, Sweden) on a fast protein liquid chromatography (FPLC), which was equilibrated with 0.05 M pyridine-acetate buffer (pH 6.0), and eluted with a gradient of 0 to 1.0 M ammonium acetate in a total volume of 50 ml of the starting buffer. The purified parasin I-producing protease was subjected to N-terminal sequence analysis by automatic Edman degradation on a gas-phase sequencer, Model 477A (Applied Biosystems, Foster City, CA).

A non-aspartyl protease was also purified from the mucus of normal catfish and analyzed by using the same methods as above except for the following modification. Because the mucus extracts of normal catfish did not show the parasin I-producing activity when incubated with histone H2A, the latent parasin I-producing proteolytic activity of each fraction (1 ml), which was collected during the protease purification from the mucus of normal catfish, was assessed after addition of the pepstatin A flow-through fraction (5 μg of protein) prepared from the mucus of wounded catfish.

cDNA cloning of the gene encoding the purified protease

A cDNA encoding the purified protease was isolated from a catfish skin cDNA library, which was constructed with a Uni-ZAP XR cDNA library kit (Stratagene, La Jolla, CA) according to the manufacturer's procedure. The library was screened with a 1641-nt probe specific to the purified protease, which was generated by 3′ RACE (3′ RACE PCR kit, Life Technologies, Rockville, MD). For the RACE procedure, a PCR primer (5′ ATHCCNYTNAARAARTT 3′, where R=A, G; Y=C, T; H=A, C, T; N=A, C, G, T) was designed based on the amino acid sequence (residues 4 to 9) of the purified protease from normal catfish and was used as a gene-specific sense primer. The nucleotide sequence of the selected clones was determined on both strands by using universal and sequence-specific primers on an automated DNA sequencer (ABI

prism model 377, Perkin Elmer, Foster City, CA).

Purification and characterization of the peptide cleaved off from histone H2A by the purified protease

Histone H2A (100 μg) was incubated for 1 h at room temperature with the protease purified from the mucus of wounded catfish (3 μg) in 100 μl of 20 mM sodium phosphate (pH 6.0) containing 50 mM NaCl. The reaction mixture was analyzed on a Waters high-pressure liquid chromatography system equipped with a C_{18} reversed-phase column (3.9×300 mm, Delta Pak, Millipore, Milford, MA). A linear gradient of 0% to 35% acetonitrile in 0.1% trifluoroacetic acid was established over a period of 1 h at a flow rate of 1 ml/min. Fractions corresponding to each peak were lyophilized, resuspended in water, and analyzed by tricine SDS-PAGE. We examined the antimicrobial activity of each peak by the radial diffusion assay on a *Bacillus subtilis* lawn as described by Lehrer et al. (18). The molecular size of the processed parasin I-like peptide was determined by matrix-associated laser desorption ionization mass spectroscopy (Kratos Kompact MALDI, Manchester, England). The amino acid sequence was determined by automatic Edman degradation as described before.

Antibody preparation

A mouse monoclonal antibody to histone H2A (BWA3) was obtained from Monestier et al. (19). We purchased a rabbit polyclonal antibody to acetylated ($Ac\text{-}Lys^5$) histone H2A (Serotec, Oxford, England) and a rabbit polyclonal antibody to human cathepsin D (Upstate Biotechnology, Lake Placid, NY). The antibody to parasin I was elicited in rabbits by injection of synthetic parasin I after conjugation via an added C-terminal cysteine residue to keyhole limpet hemocyanin (Pierce, Rockford, IL) as described by Kim et al. (17). Antibody specificities were determined by competitive ELISA as described by Monestier et al. (20). In the case of the anti-parasin I antibody, parasin I (1 μM) was used as a coated antigen, and 0 to 10 μM histone H2A or parasin I was used as competitors in the fluid phase. IC_{50} values are defined as the amount (expressed in μM) of competitor necessary to inhibit maximal antibody binding by 50%.

Immunohistochemistry

Skin samples taken from both normal and wounded catfishes were placed in 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS), incubated overnight at 4°C, and embedded in paraffin. Serial cross sections (4 μm) of the embedded catfish skin were deparaffinized, treated with 0.3% hydrogen peroxide in methanol for 30 min, and washed extensively, first with water and then with PBS. We performed immunostaining by using a biotin-avidin-peroxidase method (Vectastain ABC Elite kit, Vector, Burlingame, CA) according to the manufacturer's protocol. Briefly, the sections were incubated with 1% normal blocking serum for 20 min at room temperature and then incubated for 1 h with the following antibodies: anti-unacetylated histone H2A (20 μg/ml), anti-acetylated histone H2A (1:3000 dilution), anti-parasin I (1:1000 dilution), and anti-cathepsin D (1:1000 dilution). Sections were developed with diaminobenzidine for 10 min and then counterstained with Mayer's hematoxylin, dehydrated, and mounted. Reagents for the hematoxylin and eosin stain and the periodic acid-Schiff (PAS) stain were obtained from Poly Scientific (Bay Shore, NY). We performed control incubations by

using equivalent dilutions of rabbit preimmune serum as a primary antibody.

Preparation of peptide samples from the skin mucus of fish

Skin mucus (50 mg of protein) was collected from wounded catfish, eel (*Anguilla japonica*), loach (*Misgurnus anguillicandatus*), and rainbow trout (*Oncorhynchus mykiss*). The mucus samples were homogenized separately in 100 ml of extraction medium (0.2 M sodium acetate, pH 7.0; 0.2% Triton X-100; and 50 μM pepstatin A). After centrifugation, the supernatants were collected and subjected to reversed-phase concentration with Sep-Pak C_{18} cartridges (Millipore) as described by Park et al. (5). The prepared peptide samples were analyzed by Western blotting.

Western blot analysis

For immunoblot analysis of cathepsin D and parasin I, samples containing an equal amount of protein were separated by either 10.0% SDS-PAGE or 16.5% tricine SDS-PAGE and blotted onto nitrocellulose membranes (0.2 μm pore size, Bio-Rad, Hercules, CA) by semidry electrophoretic transfer. Nonspecific protein binding sites were blocked with 25 mM Tris-HCl, pH 7.2; 150 mM NaCl; and 0.05% Tween 20 (TBS-T) containing 3% bovine serum albumin (BSA). The membranes were incubated with either anti-parasin I antibody (1:2000 dilution) or anti-cathepsin D antibody (1:2000 dilution) for 1 h at room temperature in TBS-T containing 0.5% BSA. The second antibody was an anti-rabbit IgG conjugated with horseradish peroxidase (Amersham, Aylesbury, England). Immunoreactivity was visualized by enhanced chemiluminescence (ECL kit, Amersham).

Antimicrobial assays

Aeromonas salmonicida was cultured in trypticase soy broth at 26°C. *Cytophaga aquatilis* and *Yersinia ruckeri* were cultured in nutrient broth at 26°C. *Edwardsiella ictaluri* and *Lactococcus garvieae* were cultured in brain heart infusion medium at 26°C and 30°C, respectively. The minimal inhibitory concentrations (MICs) against the fish-specific pathogens listed above were determined by incubating approximately 5×10^4 colony forming units with serial dilutions of synthetic parasin I in each well of a static 96-well microtiter plate (Corning, Acton, MA) as described by Park et al. (21). The inhibition of growth was determined by measuring the absorbance at 620 nm with a Model 550 Microplate Reader (Bio-Rad). The lowest concentration of peptide that completely inhibited bacterial growth was defined as the MIC.

RESULTS

Cathepsin D is responsible for the proteolytic cleavage of histone H2A to yield parasin I

As a preliminary experiment, we assessed the specific proteolytic activity that converts histone H2A to parasin I in the mucus extracts from both normal and wounded catfishes. The mucus

extracts of normal catfish did not show parasin I-producing activity, however, the mucus extracts from wounded catfish had a parasin I-producing activity (pH range, 5.0 to 6.0, Fig. 1A). Enzyme inhibition studies showed that the production of parasin I from histone H2A by an activity in the mucus extracts of wounded catfish was abolished completely by pretreatment of the mucus with pepstatin A, a strong aspartic protease inhibitor (Fig. 1B). However, the serine protease inhibitor PMSF, cysteine protease inhibitor E-64, and metalloprotease inhibitor EDTA had no effect on the parasin I-producing activity. These results indicate that an aspartic protease(s) is involved in the production of parasin I from histone H2A in the mucus of wounded catfish. Therefore, we sought to purify these aspartic protease(s) from the mucus of wounded catfish by using a pepstatin A-agarose column. The fraction from the pepstatin A-agarose column that showed parasin I-producing activity was separated further into a single protein band of ~37 kDa by chromatography on an FPLC Mono Q column (Fig. 2A, B).

This purified enzyme was subjected to automated Edman degradation for amino acid sequence analysis, which allowed identification of the N-terminal 18 amino acid residues (Fig. 2C). A search of the National Center for Biotechnology Information (NCBI) data bank through the BLAST network service showed that the amino acid sequence from the amino terminus of the parasin I-producing enzyme closely matched the N-terminal sequence of cathepsin D, especially that of icefish cathepsin D (22). The proteolytic activity of cathepsin D was tracked throughout the purification by the presence of the processed parasin I-like peptide (as determined by tricine SDS-PAGE) and the antimicrobial activity (Fig. 3). The amino acid sequence of the processed parasin I-like peptide, which was determined by automatic Edman degradation, was SGRGKQGGKARAKATRSS and, in repeated mass analysis, the peptide was found to have a mean mass value of 1932.5 Da. The amino acid sequence of the processed parasin I-like peptide was different from that of the natural parasin I in two residues because two residues in parasin I (K^1 and V^{10}) are different from those of calf thymus histone H2A (S^1 and A^{10}). This difference resulted in a different mass between the processed parasin I-like peptide (1932.5 Da) and the natural parasin I (2000.4 Da). On the basis of the above results, we concluded that cathepsin D cleaved the Ser^{19}-Arg^{20} bond of histone H2A to produce parasin I.

Procathepsin D is activated to mature cathepsin D by a metalloprotease that is induced on injury

Cathepsin D is known to be expressed constitutively in almost all cells (23), except in particular forms of cancer (24) and in oocyte maturation of egg-laying animals (25). Therefore, the observation that parasin I production is induced by epidermal injury (5) implies the presence of the responsible enzyme(s) in the mucus of wounded catfish that is subject to regulation. We hypothesized that cathepsin D might be secreted to the mucus as a latent proform and then activated to a mature form by an enzyme(s) induced in the mucus of wounded catfish. To address our proposed hypothesis, we attempted to isolate procathepsin D from the mucus of normal catfish by using the same methods as described for the mucus from wounded catfish. The pepstatin A-bound fractions obtained from the mucus of normal catfish did not show parasin I-producing activity. However, the activity was generated when the pepstatin A flow-through fraction prepared from the mucus of wounded catfish were added to the pepstatin A-bound fractions. Fractions showing parasin I-producing activity with addition of the pepstatin A flow-through fraction prepared from the mucus of wounded catfish were purified further by FPLC on

a Mono Q column, and a 40 kDa protein was purified (Fig. 4). This purified protein was subjected to automated Edman degradation for amino acid sequence analysis, and the first 24 amino acid residues were identified as LVRIPLKKFRSIRRTMSDSGRAVE. A cDNA encoding the 40 kDa protein was cloned by 3' RACE and cDNA library screening. DNA sequence analysis of the 1749-bp cDNA (GenBank accession no. AF396662) showed an open reading frame that encodes a 395-residue polypeptide that consisted of a putative signal peptide of 18 amino acids, a prosequence extending 43 amino acids, and a mature enzyme of 334 amino acids. A search of the NCBI data bank through the BLAST network service identified the purified protein as procathepsin D. Western blot analysis showed that the 40 kDa procathepsin D was processed to 37 kDa mature cathepsin D by the pepstatin A flow-through fraction prepared from the mucus of wounded catfish (Fig. 5). The processing of procathepsin D to the mature cathepsin D was also examined with the pepstatin A flow-through fraction prepared from the mucus of normal catfish, but the cathepsin D-activating enzyme activity was not detected (data not shown). Furthermore, the cathepsin D-activating enzyme activity was inhibited only by EDTA, which suggests that the cathepsin D-activating enzyme induced in the mucus of wounded catfish is a metalloprotease (Fig. 5). From these results, we concluded that parasin I was produced from histone H2A by two steps: (i) cathepsin D was secreted to the mucus as a proform, and (ii) a metalloprotease induced on injury cleaves procathepsin D to form active cathepsin D, which consequently cleaves the Ser^{19}-Arg^{20} bond of histone H2A.

Parasin I is produced on the mucosal surface upon epidermal injury

Immunohistochemical experiments were performed to determine the histological location of histone H2A, parasin I, and cathepsin D in cross sections of normal (Fig. 6A–G) and wounded (Fig. 6H) catfish skins. To distinguish the histone H2A present in cytoplasm from that in the nucleus, we used two specific antibodies, anti-acetylated ($Ac\text{-}Lys^5$) histone H2A and anti-unacetylated histone H2A (17). The monoclonal antibody to unacetylated histone H2A (BWA3) stained the cytoplasm of epithelial mucous cells (Fig. 6D), whereas the anti-acetylated histone H2A stained only the nucleus (Fig. 6E). The catfish skin section that had been subjected to PAS-staining revealed that cells that were immunopositive for unacetylated histone H2A were also the PAS-positive cells responsible for the generation of mucus (Fig. 6B). Incubation of the skin sections from normal (Fig. 6G) and wounded (Fig. 6H) catfishes with a polyclonal antibody to parasin I showed strong immunoreactivity only at the mucosal surface of the wounded skin. This finding indicates that parasin I was produced on the mucosal surface on epidermal injury. The specificities of BWA3 and the polyclonal antibody to parasin I were confirmed further with a competitive ELISA assay (Fig. 7). This assay showed that the binding of BWA3 to histone H2A was not inhibited by parasin I (IC_{50} >10 μM), and the binding of the polyclonal antibody to parasin I was only slightly inhibited by histone H2A (IC_{50} =7.8 μM). Control sections of catfish skin incubated with preimmune serum showed no immunoreactivity (Fig. 6C). A polyclonal antibody specific to human cathepsin D localized the immunoreactivity of procathepsin D to the cytoplasm of epithelial mucous cells, the same cells that contained unacetylated histone H2A (Fig. 6F). Given that cathepsin D was isolated as a proenzyme from the mucus of normal catfish, our results indicate that the cathepsin D immunoreactivity in epithelial mucous cells of normal catfish resulted from procathepsin D, although the antibody cannot distinguish procathepsin D from cathepsin D.

Parasin I is also present in the mucus of other fish species

We performed Western blot analysis to determine whether parasin I could be detected in other fish species. Skin mucus was prepared from wounded catfish, eel, loach, and rainbow trout and was partially purified by reversed-phase concentration. Western blot analysis identified in the mucus of all fish tested an immunoreactive peptide that corresponded to purified parasin I (Fig. 8).

Parasin I shows good antimicrobial activity against fish-specific pathogens

Five fish-specific pathogens (Table 1) were used to test the bactericidal activity of synthetic parasin I: *A. salmonicida* (isolated from the skin of salmon), *C. aquatilis* (isolated from the gills of diseased salmon), *Y. ruckeri* (isolated from the rainbow trout with red mouth disease), *E. ictaluri* (isolated from a channel catfish), and *L. garvieae* (isolated from the kidney of diseased yellowtail). Results of the antimicrobial assays showed that parasin I is active against both Gram-negative and Gram-positive fish-specific pathogens.

DISCUSSION

In this study, we have demonstrated that the antimicrobial peptide parasin I is generated from unacetylated histone H2A in catfish skin mucosa by the action of cathepsin D, which is secreted to the mucosal surface as an inactive proenzyme (procathepsin D) and is activated to the mature enzyme by a metalloprotease induced in response to epidermal injury. Cathepsin D generates parasin I from histone H2A by cleaving specifically the Ser^{19}-Arg^{20} bond of histone H2A, as confirmed by structural and immunological analyses of the processed peptide. The report that rat epidermal cathepsin D hydrolyzes histone H2A selectively at pH 6 (26) also supports our data. Cathepsin D is a lysosomal aspartic protease that is synthesized as an inactive proenzyme (procathepsin D) and is activated most likely in the lysosome (23). It has been reported that 5% to 15% of procathepsin D escapes targeting to the lysosome and is secreted from cells. It has been detected in human milk (27) and urine (28) as well as in bovine milk (29). We purified procathepsin D from the mucus of normal catfish and measured its proteolytic activation to mature cathepsin D by Western blot analysis (Fig. 5). The 40 kDa procathepsin D was processed to 37 kDa mature cathepsin D by an enzyme present in the mucus of wounded catfish. The incomplete conversion to cathepsin D in our assay may be due to insufficient quantities of the responsible enzyme or the suboptimal reaction conditions. It has been suggested that an unknown cysteine protease is responsible for the formation of mature cathepsin D in the lysosome (30), but little is known about the activation of secreted procathepsin D by extracellular proteases. Our protease inhibition assay revealed that the cathepsin D-activating enzyme induced in the mucus of wounded catfish is a metalloprotease (Fig. 5). This discrepancy may be accounted for by the fact that the enzyme profiles are different in the skin mucus and in the lysosome. Fish mucus has been reported to contain a variety of enzyme activities (31), including metalloproteases (32, 33), whereas no metallo-endopeptidase is known to exist in the lysosome (34). In vertebrates, metalloproteases, which are expressed or released in response to injury, disease, or inflammation, are typically associated with the immune response and tissue repair (35). Among them, matrilysin (matrix metalloproteinase 7), the expression of which is induced by exposure to bacteria, has been reported to function in the intestinal mucosal defense

by regulating the activity of defensins (36). The inducible mechanism of the parasin I-producing activity in this study is in agreement with the findings of Park et al. that parasin I is found only in the skin secretions of wounded catfish and not in unwounded catfish (5). Cathepsin D is thought to function primarily in the normal degradation of cellular and phagocytosed proteins in the lysosome (23). It has also been postulated to play a role in antigen and prohormone processing (37, 38), and its overexpression and increased secretion have been associated with the malignant behavior of human breast cancer (39). Our results suggest that the role of cathepsin D may be extended to the innate host defense system, specifically by processing histone H2A to yield the antimicrobial peptide parasin I.

To elucidate the mechanism of parasin I generation in catfish skin mucosa, we performed immunohistochemical analyses with skin sections from both normal and wounded catfishes. Immunohistochemical data indicate that unacetylated histone H2A, the precursor of parasin I, and procathepsin D are present in the epithelial mucous cells (Fig. 6D, F). This finding suggests that they are secreted to the mucosal surface in a way similar to other mucous substances. It appears that a large amount of unacetylated histone H2A is available in the cytoplasm of epithelial mucous cells. This results in part from the rapid regeneration of the skin mucosa and the active transcription in epithelial mucous cells, which are reflected in the high expression of histone proteins. Of the total histone H2A synthesized in the epithelial mucous cells, a limited amount moves into the nucleus and is acetylated (40), whereas the excess unacetylated histone H2A accumulates in the cytoplasm and eventually is secreted to the mucosal surface. The immunoreactivity of unacetylated histone H2A and procathepsin D did not change on injury (data not shown), whereas the immunoreactivity of parasin I at the mucosal surface of the skin was increased greatly in response to injury (Fig. 6G, H). This finding further confirms the existence of an inducible mechanism in parasin I production, which is mediated by the activation of procathepsin D secreted to the mucosal surface. Purification of the responsible enzyme, a cathepsin D-activating metalloprotease, is in progress to characterize further this inducible mechanism. Using recombinant catfish procathepsin D as a substrate, we plan to identify the metalloprotease in the mucus of wounded catfish that processes procathepsin D to cathepsin D.

Of note is the finding that the immunoreactivity of parasin I is extracellular on the mucosal surface. This indicates that parasin I coats the mucosal surface, where it may contribute to the establishment of a local antimicrobial milieu. Fish outer skin that covers the body surface consists mainly of skin mucus and epidermal cell layers. Because this tissue serves as the primary wall between the internal and external environments, it contributes significantly to physical and physiological protection against unfavorable changes, such as environmental stresses and pathogen infections (41). In those fish in which scalation is reduced or absent, copious amounts of mucus are produced to compensate for the weak mechanical barrier (42). It also has been reported that the loss of skin mucus results in an increased mortality due to infection (43), which suggests that the skin mucus prevents colonization by microorganisms. In this respect, the localization of parasin I in the skin mucosa of the catfish may be highly significant, because parasin I provides an antimicrobial barrier against microbial invasion on the skin mucosal surface. This notion is supported further by our observation that parasin I showed good antimicrobial activity against fish-specific pathogens (Table 1).

The conservation of the amino acid sequence of histone H2A (44) and cathepsin D (22, 45) in

the fish phylum suggests that this parasin I sequence constitutes a biologically important part of the molecule that confers an advantage during selection and evolution in fish. Whether parasin I is ubiquitous in the skin mucosa of other fish is not yet clear. However, we confirmed by Western blot analysis the presence of parasin I in the mucus of eel, loach, and rainbow trout, in which scalation is reduced or absent (Fig. 8). Taken together, our results suggest that cathepsin D is responsible for the generation of parasin I from histone H2A on epidermal injury and that parasin I represent an important innate antimicrobial defense against invading microorganisms in the skin mucosa of fish.

ACKNOWLEDGMENTS

We thank Marc Monestier for providing monoclonal antibody BWA3 to histone H2A. This work was supported in part by grants from the Korea Science and Engineering Foundation (R01-1999-1-00109) and the National Research Laboratory Program (2000-N-NL-01-C-160) of the Ministry of Science and Technology.

REFERENCES

1. Ellis, A. E. (1982) Differences between the immune mechanisms of fish and higher vertebrates. In *Microbial Disease of Fish* (Robert, R., ed.), Academic Press: London, U.K.; pp. 1–30

2. Bly, J. E., and Clem, L. W. (1991) Temperature-mediated processes in teleost immunity: in vitro immunosupression induced by in vivo low temperature in cannel catfish. *Vet. Immunol. Immunopathol.* **28**, 365–377

3. Hancock, R. E. W., and Lehrer, R. I. (1998) Cationic peptides: a new source of antibiotics. *Trends Biotechnol.* **16**, 82–88

4. Cole, A. M., Weis, P., and Diamond, G. (1997) Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. *J. Biol. Chem.* **272**, 12008–12013

5. Park, I. Y., Park, C. B., Kim, M. S., and Kim, S. C. (1998) Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*. *FEBS Lett.* **437**, 258–262

6. Patrzykat, A., Zhang, L., Mendoza, V., Iwama, G. K., and Hancock, R. E. W. (2001) Synergy of histone-derived peptides of coho salmon with lysozyme and flounder pleurocidin. *Antimicrob. Agents Chemother*. **45**, 1337–1342

7. Boman, H. G. (1995) Peptide antibiotics and their role in innate immunity. *Annu. Rev. Immunol.* **13**, 61–92

8. Hancock, R. E. W., and Scott, M. G. (2000) The role of antimicrobial peptides in animal defenses. *Proc. Natl. Acad. Sci. USA* **97**, 8856–8861

9. Hunter, K. M., and Bevins, C. L. (1999) Antimicrobial peptides as mediators of epithelial host defense. *Pediatr. Res*. **45**, 785–794

10. Murphy, C. J., Foster, B. A., Mannis, M. J., Selsted, M. E., and Reid, T. W. (1993) Defensins are mitogenic for epithelial cells and fibroblasts. *J. Cell Physiol.* **155**, 408–413

11. Gallo, R. L., Ono, M., Povsic, T., Page, C., Eriksson, E., Klagsbrun, M., and Bernfield, M. (1994) Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. *Proc. Natl. Acad. Sci. USA* **91**, 11035–11039

12. Huang, H. J., Ross, C. R., and Blecha, F. (1997) Chemoattractant properties of PR-39, a neutrophil antibacterial peptide. *J. Leukoc. Biol.* **61**, 624–629

13. Yeoman, L. C., Olson, M. O., Sugano, N., Jordan, J. J., Taylor, D. W., Starbuck, W. C., and Busch, H. (1972) Amino acid sequence of the COOH-terminal portion of the arginine-lysine-rich histone of calf thymus. *J. Biol. Chem.* **247**, 6018–6023

14. Hirsch, J. G. (1958) Bactericidal action of histone. *J. Exp. Med.* **108**, 925–944

15. Hiemstra, P. S., Eisenhauer, P. B., Harwig, S. S. L., van den Barselaar, M. T., van Furth, R., and Lehrer, R. I. (1993) Antimicrobial proteins of murine macrophages. *Infect. Immun*. **61**, 3038–3046

16. Rose, F. R., Bailey, K., Keyte, J. W., Chan, W. C., Greenwood, D., and Mahida, Y. R. (1998) Potential role of epithelial cell-derived histone H1 protein in innate antimicrobial defense in the human gastrointestinal tract. *Infect. Immun*. **66**, 3255–3263

17. Kim, H. S., Yoon, H., Minn, I., Park, C. B., Lee, W. T., Zasloff, M., and Kim, S. C. (2000) Pepsin-mediated processing of the cytoplasmic histone H2A to strong antimicrobial peptide buforin I. *J. Immunol.* **165**, 3268–3274

18. Lehrer, R. I., Rosenman, M., Harwig, S. S. L., Jackson, R., and Eisenhauer, P. (1991) Ultrasensitive assays for endogenous antimicrobial polypeptides. *J. Immunol. Methods* **137**, 167–173

19. Monestier, M., Fasy, T. M., Losman, M. J., Novick, K. E., and Muller, S. (1993) Structure and binding properties of monoclonal antibodies to core histones from autoimmune mice. *Mol. Immunol*. **30**, 1069–1075

20. Monestier, M., Decker, P., Briand, J. -P., Gabriel, L., and Muller, S. (2000) Molecular and structural properties of three autoimmune IgG monoclonal antibodies to histone H2B. *J. Biol. Chem*. **275**, 13558–13563

21. Park, C. B., Yi, K. S., Matsuzaki, K., Kim, M. S., and Kim, S. C. (2000) Structure-activity analysis of buforin II, a histone H2A-derived antimicrobial peptide: the proline hinge is

responsible for the cell-penetrating ability of buforin II. *Proc. Natl. Acad. Sci. USA* **97**, 8245–8250

22. Capasso, C., Lees, W. E., Capasso, A., Scudiero, R., Carginale, V., Kille, P., Kay, J., and Parisi, E. (1999) Cathepsin D from the liver of the antarctic icefish *Chionodraco hamatus* exhibits unusual activity and stability at high temperatures. *Biochim. Biophys. Acta*. **1431**, 64–73

23. Conner, G. E. (1998) Cathepsin D. In *Handbook of Proteolytic Enzymes* (Barrett, A. J., Rawlings, N. D., and Woessner, J. F., eds.) Academic Press: London, U.K.; pp. 828–836

24. Garcia, M., Platet, N., Liaurdet, E., Laurent, V., Derocq, D., Brouillet, J. P., and Rochefort, H. (1996) Biological and clinical significance of cathepsin D in breast cancer metastasis. *Stem Cells* **14**, 642–650

25. Brooks, S., Tyler, C. R., Carnevali, O., Coward, K., and Sumpter, J. P. (1997) Molecular characterization of ovarian cathepsin D in the rainbow trout, *Oncorhynchus mykiss*. *Gene* **201**, 45–54

26. Harvima, R. J., Yabe, K., Fraki, J. E., Fukuyama, K., and Epstein, W. L. (1988) Hydrolysis of histones by proteinases. *Biochem. J.* **250**, 859–864

27. Vetvicka, V., Vagner, J., Baudys, M., Tang, J., Foundling, S. I., and Fusek, M. (1993) Human breast milk contains procathepsin D: detection by specific antibodies. *Biochem. Mol. Biol. Int.* **30**, 921–928

28. Zuhlsdorf, M., Imort, M., Hasilik, A., and von Figura, K. (1983) Molecular forms of beta-hexosaminidase and cathepsin D in serum and urine of healthy subjects and patients with elevated activity of lysosomal enzymes. *Biochem. J.* **213**, 733–740

29. Larsen, L. B., Boisen, A., and Petersen, T. E. (1993) Procathepsin D cannot autoactivate to cathepsin D at acid pH. *FEBS Lett*. **319**, 54–58

30. Samarel, A. M., Ferguson, A. G., Decker, R. S., and Lesch, M. (1989) Effects of cysteine protease inhibitors on rabbit cathepsin D maturation. *Am. J. Physiol.* **257**, C1069–C1079

31. Yano, T. (1996) The nonspecific immune system: Humoral defense. In *The Fish Immune System* (Iwama, G., and Nakanishi, T., eds.) Academic Press: London, U.K.; pp. 105–157

32. al-Hassan, J. M. (1990) Diabetic ulcer healing preparations from the skin of the Arabian Gulf catfish (*Arius bilineatus* Val.): a novel and effective treatment. *Int. J. Tissue React.* **12**, 121–135

33. Brun, N. T., Ross, N. W., and Boghen, A. D. (2000) Changes in the electrophoretic profiles of gill mucus proteases of the eastern oyster *Crassostrea virginica* in response to infection by the turbellarian *Urastoma cyprinae*. *J. Invertebr. Pathol.* **75**, 163–170

34. Erickson, A. H. (1989) Biosynthesis of lysosomal endopeptidases. *J. Cell. Biochem.* **40**, 31–41

35. Woessner, J. F. Jr. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J.* **5**, 2145–2154

36. Wilson, C. L., Ouellette, A. J., Satchell, D. P., Ayabe, T., Lopez-Boado, Y. S., Stratman, J. L., Hultgren, S. J., Matrisian, L. M., and Parks, W. C. (1999) Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* **286**, 113–117

37. Pillai, S., and Zull, J. (1986) Production of biologically active fragments of parathyroid hormone by isolated Kupffer cells. *J. Biol. Chem.* **261**, 14919–14923

38. Guagliardi, L. E., Koppelman, B., Blum, J. S., Marks, M. S., Cresswell, P., and Brodsky, F. M. (1990) Co-localization of molecules involved in antigen processing and presentation in an early endocytic compartment. *Nature* (*London*) **343**, 133–139

39. Westley, B. R., and May, F. E. (1996) Cathepsin D and breast cancer. *Eur. J. Cancer* **32A**, 15–24

40. Schiltz, R. L., Mizzen, C. A., Vassilev, A., Cook, R. G., Allis, C. D., and Nakatani, Y. (1999) Overlapping but distinct patterns of histone acetylation by the human coactivators p300 and PCAF within nucleosomal substrates. *J. Biol. Chem.* **274**, 1189–1992

41. Suzuki, Y. (1995) Skin. In *An Atlas of Fish Histology* (Takashima, F., and Hibiya, T., eds.) John Wiley & Sons, Inc.: New York, NY; pp. 8–17

42. Alexander, J. B., and Ingram, G. A. (1992) Noncellular nonspecific defense mechanisms of fish. *Annu. Rev. Fish. Dis.* **2**, 249–279

43. Lemaitre, C., Orange, N., Saglio, P., Saint, N., Gagnon, J., and Molle, G. (1996) Characterization and ion channel activities of novel antibacterial proteins from the skin mucosa of carp (*Cyprinus carpio*). *Eur. J. Biochem*. **240**, 143–149

44. Connor, W., States, J. C., Mezquita, J., and Dixon, G. H. (1984) Organization and nucleotide sequence of rainbow trout histone H2A and H3 genes. *J. Mol. Evol.* **20**, 236–250

45. Riggio, M., Scudiero, R., Filosa, S., and Parisi, E. (2000) Sex- and tissue-specific expression of aspartic proteinases in *Danio rerio* (zebrafish). *Gene* **260**, 67–75

Received October 3, 2001; revised November 29, 2001.

Table 1

Antimicrobial activity of parasin I against fish-specific pathogens.

Strain (Gram +/-)	MIC[a] (μg/ml)
Aeromonas salmonicida *(-)*	10
Cytophaga aquatilis (-)	5
Yersinia rukeri (-)	15
Edwardsiella ictaluri (-)	10
Lactococcus garvieae (+)	10

[a]MICs were the average values obtained in triplicates on three independent measurements in three independent experiments.

Fig. 1

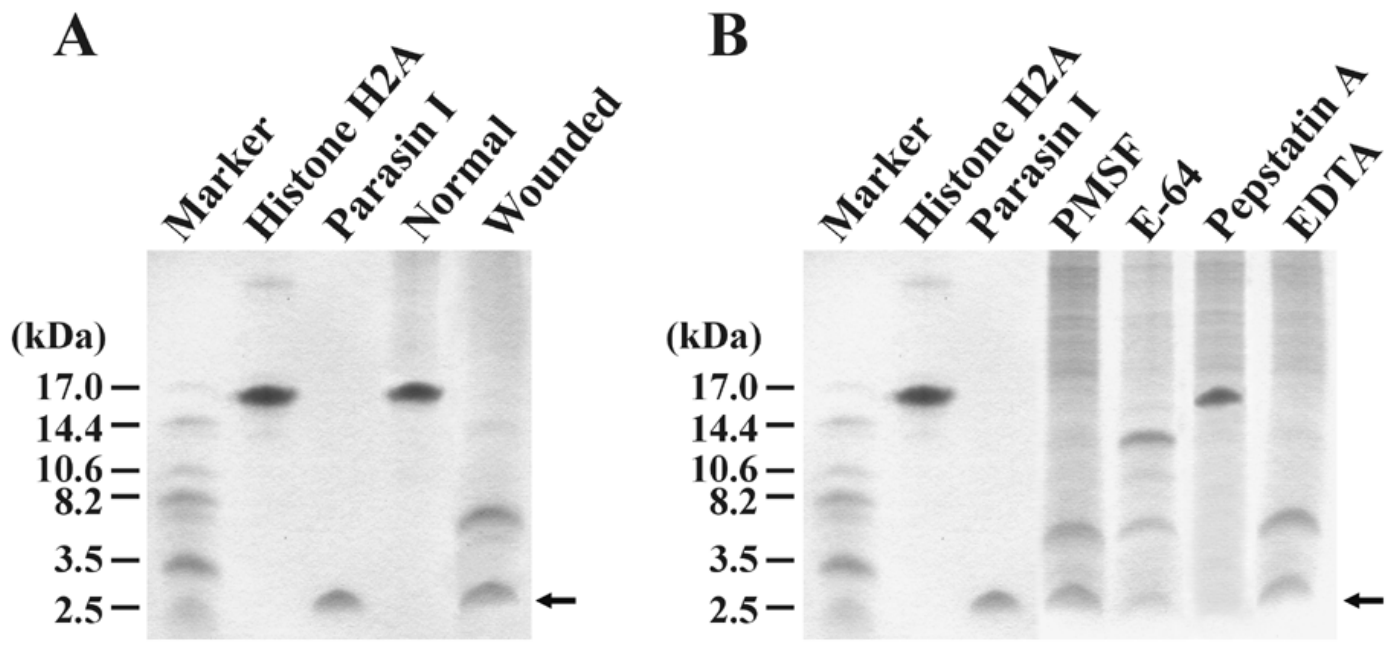
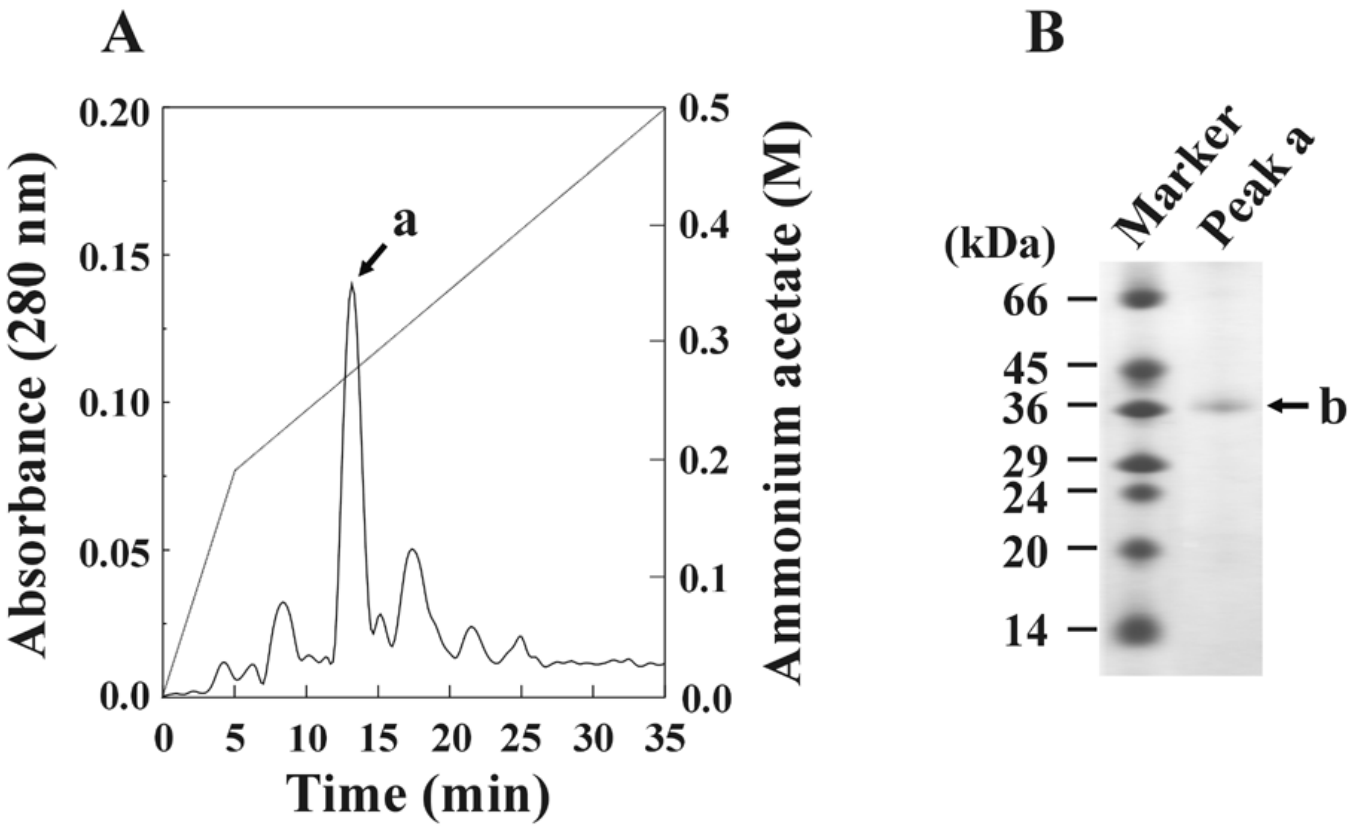


Figure 1. Proteolytic cleavage of histone H2A. A) Proteolytic activity of the mucus extracts. The mucus extracts (5 μg of protein) from either normal or wounded catfish were incubated with histone H2A (10 μg) in 50 μl of 20 mM sodium phosphate (pH 6.0) containing 50 mM NaCl. After incubation for 1 h at room temperature, the reactions were stopped by boiling for 5 min and analyzed by 16.5% tricine SDS-PAGE. The processed parasin I-like peptide is indicated by an arrow. **B)** Inhibitor sensitivity of the mucus extracts. The mucus extracts (5 μg of protein) from the wounded catfish were pre-incubated for 1 h at room temperature with each protease inhibitor: PMSF, E-64, pepstatin A, and EDTA. Histone H2A (10 μg) was then added, and the reactions were allowed to proceed for an additional hour (final reaction volume, 50 μl in reaction buffer).

Fig. 2



C

```
Catfish D       GPTPETLKNYLDAQYYGE--
Icefish D       APTPETLKNYLDAQYYGE--
Zebrafish D     DPTPETLKNYLDAQYYGE--
Trout D         GPTPETLKNFMDAQYYGE--
Human D         GPIPEVLKNYMDAQYYGE--
Mouse D         EPVSELLKNYLDAQYYGD--
Rat D           EPVSELLKNYLDAQYYGE--
                *   *  ***..******.
```

Figure 2. Purification of a specific protease from the mucus of wounded catfish. A) Chromatography on an FPLC Mono Q column of the active fractions collected from the pepstatin A-agarose affinity column. **B)** SDS-PAGE analysis of the purified specific protease. An FPLC fraction containing the parasin I-producing activity (indicated by an arrow, a) was subjected to 10.0% SDS-PAGE. The arrow (b) indicates the protease band. **C)** Alignment of the amino acid sequence of the specific protease purified from catfish. The multiple sequence alignment was obtained BY using the CLUSTAL W program with default parameters. The amino acid sequence of the specific catfish protease (designated as catfish D) is aligned with those of cathepsin Ds from icefish, zebrafish, rainbow trout, mouse, rat, and human. Asterisks and dots indicate perfect matches of amino acids and conservative substitutions, respectively.

Fig. 3

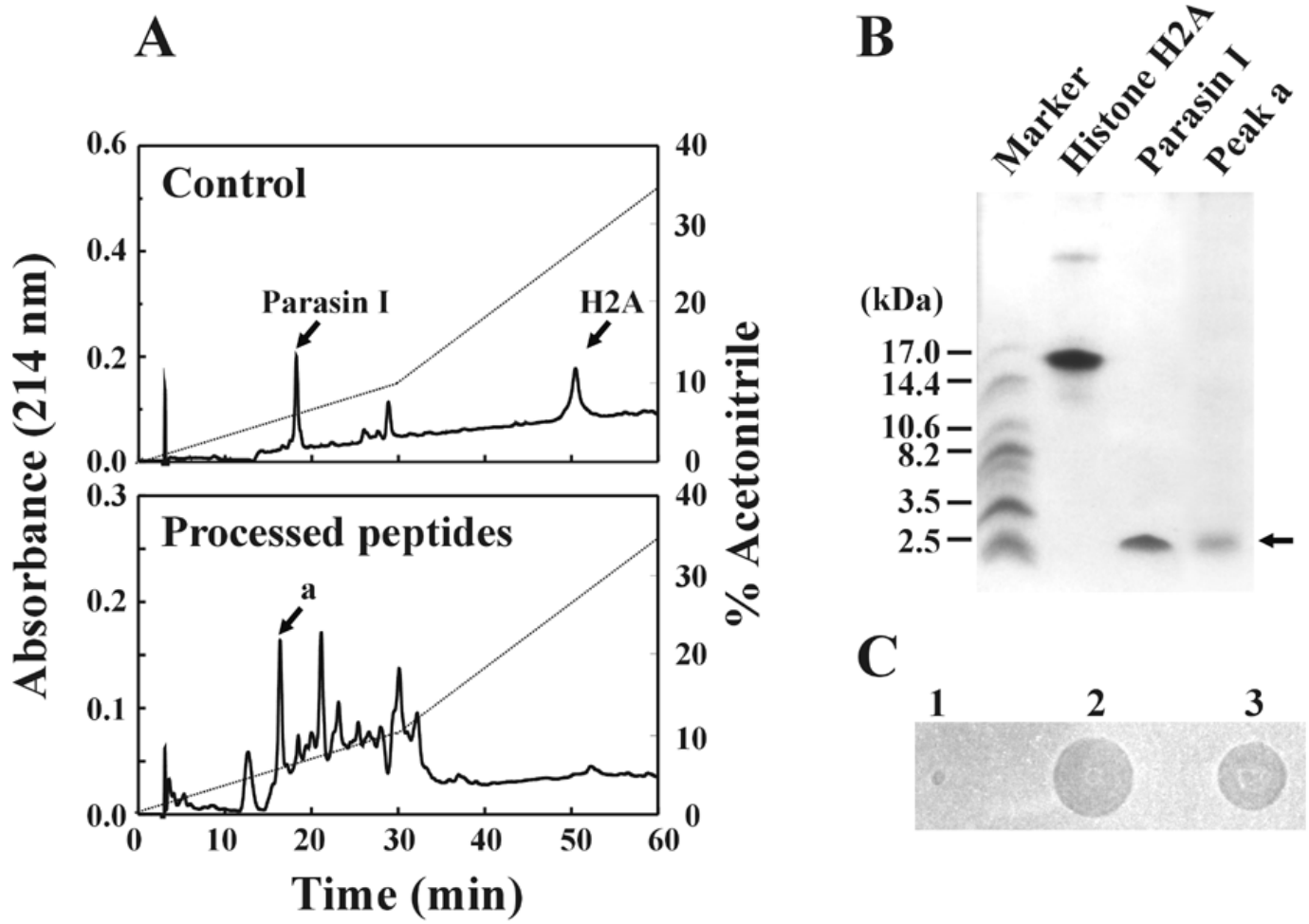


Figure 3. ***In vitro*** **production of parasin I-like peptide. A)** Reversed-phase HPLC and **(B)** 16.5% tricine SDS-PAGE analysis of histone H2A processed by catfish cathepsin D. Histone H2A and synthetic parasin I were loaded as controls. **C)** Antimicrobial analysis of the processed parasin I-like peptide. 1, 2, and 3 indicate distilled water, synthetic parasin I (10 μg/ml), and the processed parasin I-like peptide (10 μg/ml), respectively.

Fig. 4

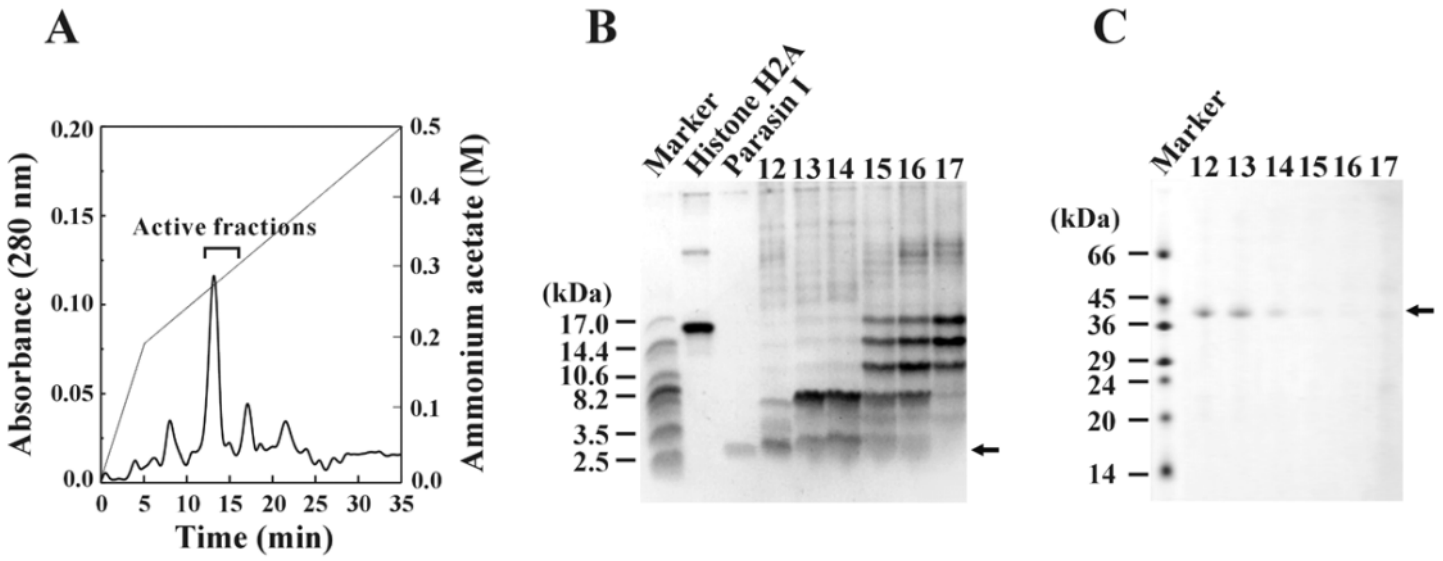


Figure 4. Purification of a specific protease from the mucus of normal catfish. A) Chromatography on an FPLC Mono Q column of the fractions showing the parasin I-producing activity on addition of the pepstatin A flow-through fraction prepared from the mucus of wounded catfish. **B)** Proteolytic cleavage of histone H2A by the fractions obtained from the Mono Q FPLC step. The parasin I-producing proteolytic activity of each fraction (1 ml) was assessed by incubating the fractions (12 to 17) with histone H2A (10 μg) after adding the pepstatin A flow-through fraction (5 μg of protein) prepared from the mucus of wounded catfish (total reaction volume, 50 μl). The processed parasin I-like peptide is indicated by an arrow. FPLC fractions numbers (12 to 17) are indicated on the top of 16.5% tricine SDS-PAGE gel. **C)** SDS-PAGE analysis of the purified specific protease. FPLC fractions containing the parasin I-producing activity (indicated by a bracket, *A*) were subjected to 10.0% SDS-PAGE. The arrow indicates the protease band.

Fig. 5

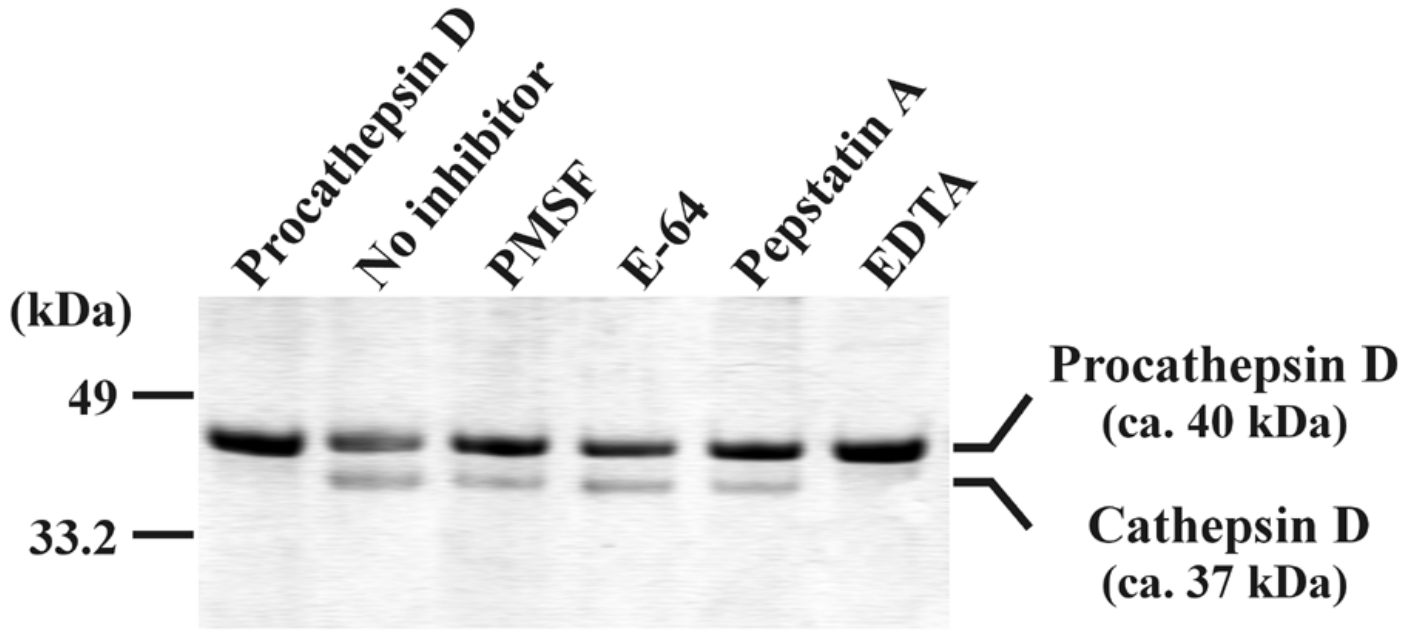


Figure 5. Cleavage of procathepsin D by pepstatin A flow-through fraction prepared from the mucus of wounded catfish. The pepstatin A flow-through fraction (5 μg of protein) prepared from the mucus of wounded catfish was pre-incubated for 1 h at room temperature with each protease inhibitor: PMSF, E-64, pepstatin A, and EDTA. Procathepsin D (2 μg) was then added, and the reactions were allowed to proceed for an additional hour (final reaction volume, 50 μl in reaction buffer containing 20 mM sodium phosphate, pH 6.0, and 50 mM NaCl). Reaction samples (10 μl) were separated by 10.0% SDS-PAGE and blotted onto a nitrocellulose membrane. The Western blot was probed with an anti-cathepsin D polyclonal antibody.

Fig. 6

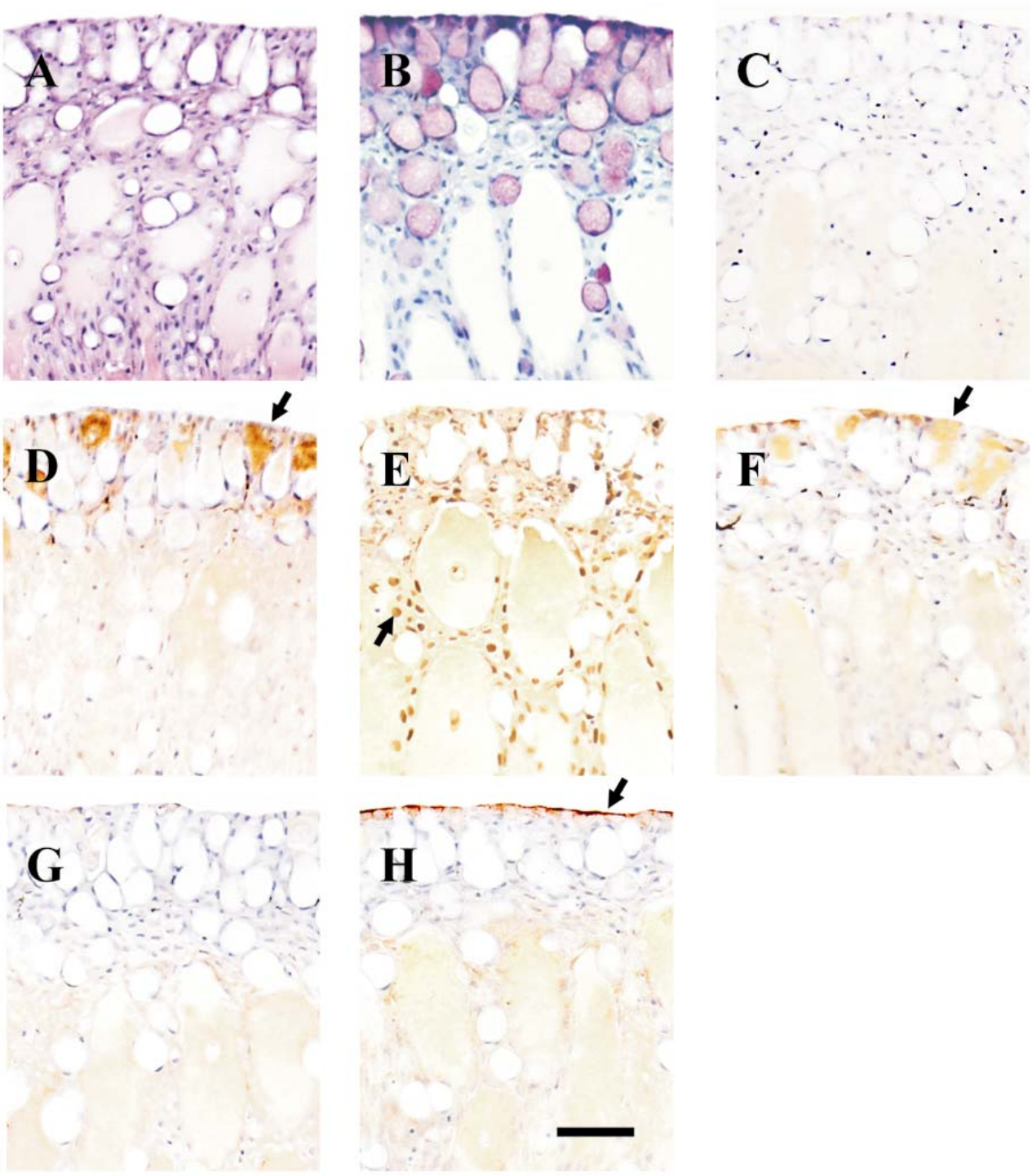


Figure 6. Immunohistochemistry of cross sections of catfish skins from normal (A-G) and wounded (H) catfishes. **A)** Hematoxylin-eosin staining was included to show skin mucosal morphology. **B)** The PAS reaction stained carbohydrate with a pink color in mucous cells. **C)** No immunoreactivity was detected with preimmune serum. **D)** Strong immunostaining for histone H2A (an arrow) was present in the cytoplasm of mucous cells from catfish skin mucosa. **E)** Strong immunostaining for acetylated (Ac-Lys5) histone H2A (an arrow) was observed in the nucleus of epithelial cells. **F)** Procathepsin D (an arrow) showed the same immunolocalization as that of histone H2A (*D*). **G)** No immunoreactivity for parasin I was detected on the mucosal surface of normal catfish skin. **H)** Intense dark staining for parasin I (an arrow) was observed on the mucosal surface of wounded catfish skin. Scale bar, 50 μm.

Fig. 7

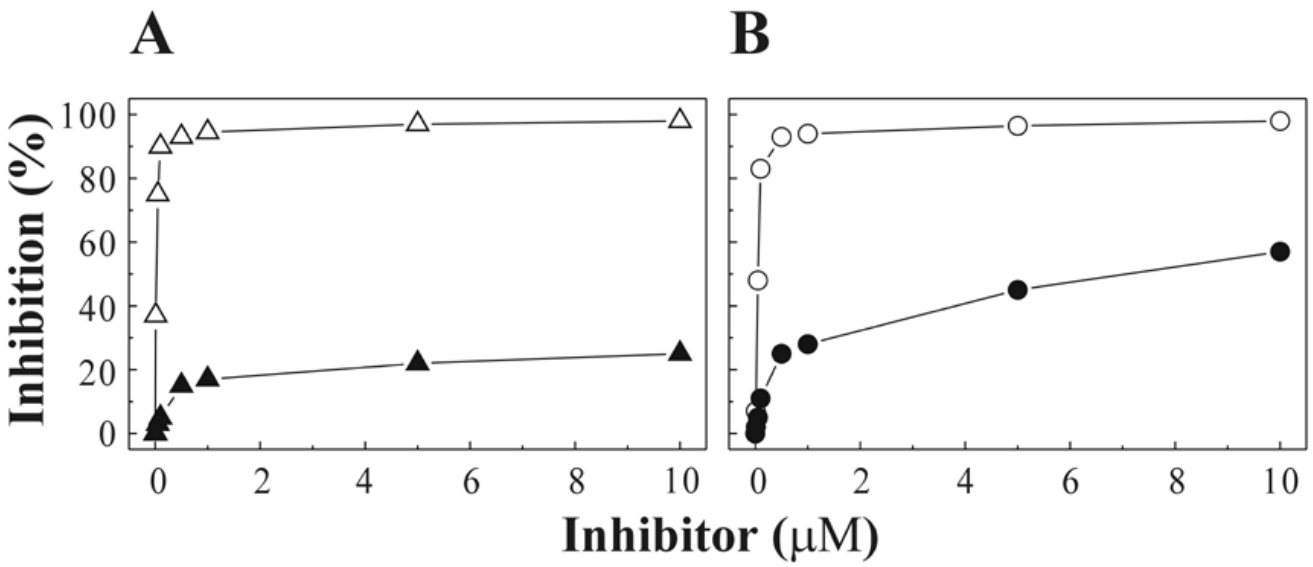


Figure 7. Specificities of anti-unacetylated histone H2A antibody and anti-parasin I antibody. Competitive ELISA assays were conducted with **(A)** anti-unacetylated histone H2A antibody (20 μg/ml) and **(B)** anti-parasin I antibody (1:1000 dilution) in the fluid phase. In **(A)**, histone H2A (1 μM) was used as a coated antigen, and 0 to 10 μM parasin I (▲) or histone H2A (△) as a competitor in the fluid phase. In **(B)**, parasin I (1 μM) was used as a coated antigen, and 0 to 10 μM histone H2A (●) or parasin I (○) as a competitor in the fluid phase. The results are expressed as percentage of inhibition of maximal antibody binding.

Fig. 8

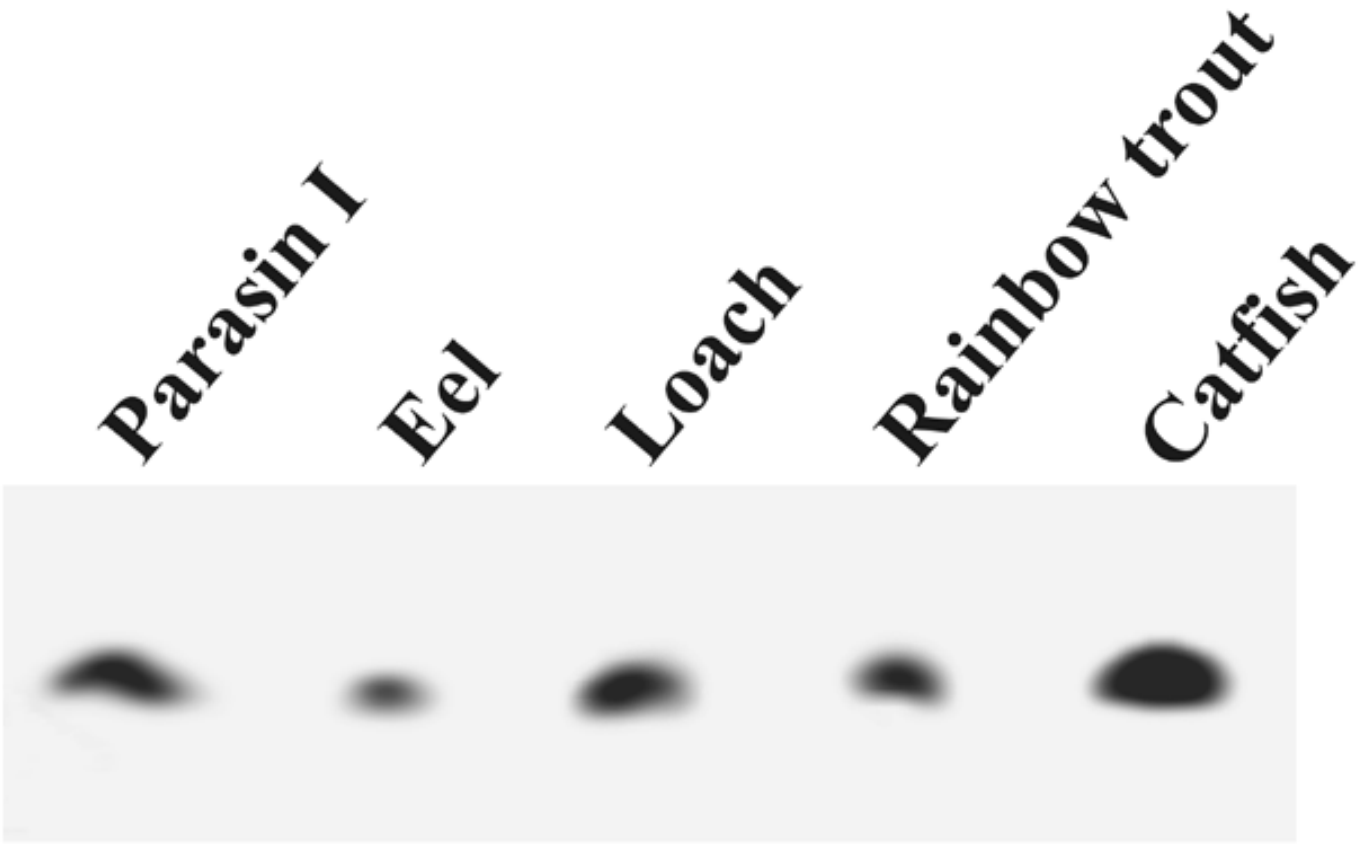


Figure 8. Western blot analysis of the skin mucous secretions from eel, loach, and rainbow trout. Skin mucus was collected and concentrated using a Sep-Pak C18 cartridge as described in Materials and Methods. The standard is 100 ng of synthetic parasin I. Each lane contains 10 μg of protein in extracts.